mol) and sodium azide (0.65 g, 0.01 mol) were heated in ethanol (50 mL)/water (20 mL) at reflux for 1 h. The reaction mixture was cooled and the precipitated solid was filtered off, washed with water, dried, and crystallized from 2-propanol (17 mL) to give the required azide, 1.05 g (71% yield), decomposing at 123–124 °C. Anal. ($C_{17}H_{17}N_3O_2$) C, H, N.

2-Amino-3-(cyclohexylmethyl)-1,4-naphthoquinone (53). 2-Azido-3-(cyclohexylmethyl)-1,4-naphthoquinone (4.26 g, 0.014 mol) in ethanol (150 mL) was hydrogenated at ambient temperature and pressure in the presence of 10% Pd/C catalyst (500 mg) for 2.5 h. The catalyst was filtered off and the filtrate was evaporated in a current of air to yield an orange solid (3.61 g), mp 100–105 °C. This was chromatographed on silica gel (50 g), eluting the desired product as a solid (2.43 g), which was further purified by crystallization from petroleum ether (150 mL) to give amine, 1.70 g (44% yield), as orange crystals, melting at 121–122 °C. Anal. ($C_{17}H_{19}NO_2$) C, H, N.

2-Cycloheptyl-3-hydroxy-5,6,7,8-tetrahydro-1,4-naphthoquinone (59). 2-Cycloheptyl-3-hydroxy-1,4-naphthoquinone (**39**; 4.0 g, 0.015 mol) in ethanol (90 mL) was hydrogenated at 70 °C (100 atm) in the presence of Raney nickel W-6[25] (6 g) for 5 h. The catalyst was filtered off and the filtrate was evaporated in a current of air to yield a brown oil (4.75 g). This was triturated with toluene (10 mL) and an insoluble purple solid (0.46 g) was filtered off. The filtrate was chromatographed on silica gel (100 g), eluting the required product with toluene as a slightly oily solid (2.6 g). Crystallization from petroleum ether afforded pure material, 1.78 g (44% yield), as orange crystals melting at 124–125 °C. Anal. ($C_{17}H_{22}O_3$) C, H.

(25) H. Adkins and H. R. Billica, *J. Am. Chem. Soc.*, **70**, 695 (1948).

Studies on Chiral Interactions of 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane and the Corresponding *N*-Hydroxy Metabolites with Cytochrome P-450

N. Peter McGraw and N. Castagnoli, Jr.*

Department of Pharmaceutical Chemistry, School of Pharmacy, Universtiy of California, San Francisco, California 94143.
Received April 3, 1980

The stereoselective pharmacological behavior and metabolism of the potent psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane have led to an investigation of the interactions of the racemic amine, its enantiomers, and the corresponding *N*-hydroxy metabolites with rabbit liver microsomal cytochrome P-450. An examination of the formation of cytochrome P-450 metabolic intermediate complexes with these species suggests that N-oxidation of the pharmacologically active (*R*)-amine is inhibited by the *S* enantiomer. Additionally, metabolic intermediate complex formation [favored by the (*R*)-amine] appears to be associated with loss of microsomal mixed function *N*-oxidase activity. The results have led to the prediction that N-hydroxylation of pure (*R*)-amine may be a qualitatively more important pathway than that observed with racemic amine even though this biotransformation may be suicidal.

The psychotomimetic properties of 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (**1**) are reported to reside in the *R* enantiomer (**1a**).[1] Drug-induced alterations of behavior in rats[2] and hyperthermia in rabbits[3] also demonstrate a chiral dependency favoring the *R* enantiomer. Studies in our laboratory have focused on the metabolic fate of amine **1** and the characterization of metabolic pathways which may be of pharmacological importance.[4-7] We have established that racemic **1** is stereoselectively metabolized by rabbits both in vivo[4] and in vitro,[6] with the *S* enantiomer being preferentially consumed. Similar results have been reported recently for rats.[8] Analysis of the enantiomeric compositions of all but one of the metabolites derived from racemic **1** showed, as expected, an *S*/*R* ratio >1.[6] N-Oxidation of racemic **1** to the hydroxylamine **2** by 100000*g* rabbit liver microsomal preparations, however, favors the *R* enantiomer.[5] This preferential N-hydroxylation of the pharmacologically active *R* enantiomer of racemic **1** prompted us to examine the interactions of racemic **1**, racemic **2**, and their individual enantiomers with rabbit liver microsomal cytochrome P-450. Binding spectra of these molecules with the oxidized form of cytochrome P-450 have been obtained by split-beam difference spectroscopy.[9] Additionally, with the aid of both difference and dual-wavelength spectroscopy[10] we have studied the possibility that these molecules, as well as other metabolites derived from **1**, may undergo oxidation to species which may bind to cytochrome P-450. These so-called metabolic intermediate complexes[11] absorb maximally near 455 nm and have been observed for a number of molecules structurally similar to **1** and **2**.[11-13] Since this complexation of cytochrome P-450 is reported to result in a reduction of mixed function oxidase activity,[11] we were particularly interested in the possible stereoselective formation of this complex and the influence of complex formation on the stereoselectivity associated with the metabolism of **1**.

Chemistry. The synthesis, resolution, and absolute configuration assignment of the parent amine **1** have been reported previously.[4] The preparations of the various

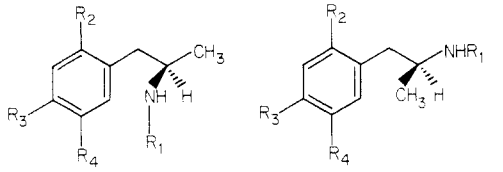
(1) A. T. Shulgin, *J. Pharm. Pharmacol.*, **25**, 271 (1973).
(2) F. Benington, R. D. Morin, J. Beaton, J. R. Smythies, and R. J. Bradley, *Nature (London), New Biol.*, **242**, 185 (1973).
(3) F. A. B. Aldous, B. C. Barass, K. Brewster, D. A. Buxton, D. M. Green, R. M. Pinder, P. Rich, M. Skeels, and K. J. Tutt, *J. Med. Chem.*, **17**, 1100 (1974).
(4) S. B. Matin, P. S. Callery, J. S. Zweig, A. O'Brien, R. Rappoport, and N. Castagnoli, Jr., *J. Med. Chem.*, **17**, 877 (1974).
(5) J. Gal, L. D. Gruenke, and N. Castagnoli, Jr., *J. Med. Chem.*, **18**, 683 (1975).
(6) N. P. McGraw, P. S. Callery, and N. Castagnoli, Jr., *J. Med. Chem.*, **20**, 185 (1977).
(7) J. S. Zweig and N. Castagnoli, Jr., *J. Med. Chem.*, **20**, 414 (1977).
(8) J. Goto, N. Goto, A Hikichi, and T. Nambara, *J. Liq. Chromatogr.*, **2**, 1179 (1979).
(9) G. J. Mannering, in "Fundamentals of Drug Metabolism and Drug Distribution", B. N. Lu Du, H. G. Mandel, and E. L. Way, Eds., Williams & Wilkins, Baltimore, 1971, pp 206–252.
(10) M. R. Franklin, *Mol. Pharmacol.*, **10**, 975 (1974).
(11) M. R. Franklin, *Hoppe-Seyler's Z. Physiol. Chem.*, **357**, 1027 (1976).
(12) M. R. Franklin, *Xenobiotica*, **4**, 133 (1974).
(13) J. Werringloer and R. W. Estabrook, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **32**, 761 (1973).

0022-2623/81/1824-0299$01.00/0

Table I. Cytochrome P-450 Binding Affinity Constants of the Enantiomers of 1 and 2

compd	concn range, μM	data points	coefficient of determination (r^2)	K_s, μM
1a	10.8-60.0	6	0.703	18
1b	1.8-60.0	7	0.997	6
2a	8.0-90.0	3	0.998	22
2b	15.0-45.0	8	0.904	68

metabolites **2**–**6**, as their racemates, also have been reported.[4]



a b

	R_1	R_2	R_3	R_4
1	H	OCH_3	CH_3	OCH_3
2	OH	OCH_3	CH_3	OCH_3
3	H	OH	CH_3	OCH_3
4	H	OCH_3	CH_3	OH
5	H	OH	CH_3	OH
6	H	OCH_3	COOH	OCH_3
7	C_6H_5COO	OCH_3	CH_3	OCH_3
8	C_6H_5CO	OCH_3	CH_3	OCH_3
9	H	H	H	H
10	OH	H	H	H

The synthesis of (*R*)- and (*S*)-1-(2,5-dimethoxy-4-methylphenyl)-2-(hydroxyamino)propane (**2a** and **2b**, respectively) was approached via hydrolysis of the corresponding (*R*)- and (*S*)-[(benzoyloxy)amino] derivatives, **7a** and **7b**. The reaction sequence first was investigated with racemic **1**. Treatment of 2 equiv of amine **1** with benzoyl peroxide in benzene at room temperature proceeded smoothly to yield 1 equiv of the benzoate salt of **1** and the desired benzoyloxy compound **7** contaminated with a small amount of 1-(2,5-dimethoxy-4-methylphenyl)-2-benzamidopropane (**8**). This side product could be separated by digestion of the crude isolate with hexane, followed by chromatography on silica. Cleavage of the *O*-benzoyl group of **7** was achieved with aqueous ethanolic HCl. The same reaction pathway led to the conversion of **1a** and **1b** to **2a** and **2b**, respectively. Enantiomeric purity of the hydroxylamines was assayed by reduction to the corresponding amines with Zn/HCl and GLC analysis of the corresponding *N*-(pentafluorobenzoyl)prolylamides by a procedure previously described.[4] Enantiomeric purity was estimated to be greater than 90% for both isomers.

Binding Studies with Oxidized Cytochrome P-450. Both enantiomers and the racemate of **1** gave typical type I binding spectra (λ_{max} 388-389 nm, λ_{min} 421–422 nm).[9] The (*S*)-*N*-hydroxy compound **2b** displayed a type II spectrum (λ_{max} 429 nm, λ_{min} 396 nm),[9] while the spectrum of the *R* enantiomer **2a** was atypical (λ_{max} 415 and 440 nm, λ_{min} 393 nm).

The apparent binding affinity constants (K_s) for the enantiomers were determined from Hofstee plots (ΔA vs. $\Delta A/[S]$)[14] by measuring the magnitude of spectral binding (ΔA) over a range of substrate concentrations [S] (Table I). The more intense maximum for **2a** (415 nm) was chosen arbitrarily for this determination. Linear regression analyses of these plots provided straight lines, the slopes

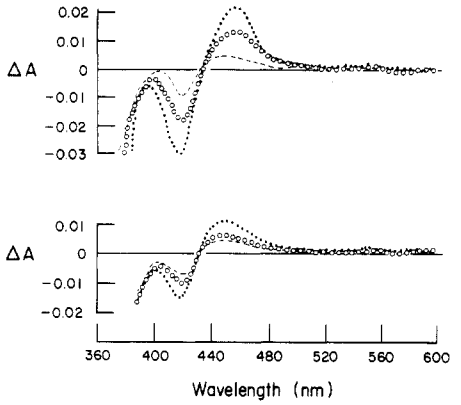


Figure 1. Difference spectra of 30 μM **1a** (upper) and 30 μM **1b** (lower) observed at 0.5 (---), 5 (O), and 15 (···) min following the addition of 0.4 mM NADPH.

of which gave estimates of $-K_s$. The coefficients of determination (r^2) of the least-squares straigth lines indicate the linearity of these plots.

The binding affinity of the *S* isomer **1b** (K_s = 6 μM) was significantly greater than that of the *R* isomer **1a** (K_s = 18 μM). The low coefficient of determination for **1a**, however, indicated that this compound does not display concentration-dependent binding as well as **1b**. The opposite stereoselectivity was observed for the binding affinities of the enantiomers of **2** (Table I). It should be noted, however, that the peculiar spectrum observed for **2a** may invalidate this comparison.

Formation of Metabolic Intermediate Complexes. Formation of cytochrome P-450 metabolic intermediate complexes derived from racemic **1** and racemic **2** and their respective enantiomers was examined by difference spectroscopy. As applied to this study, the technique involves recording the difference spectrum between two solutions which differ only by the presence of substrate in the sample cell. Complex formation is initiated by addition of NADPH to both the sample and the reference cell. In addition to the parent amine **1** and the *N*-hydroxy metabolite **2**, compounds **3**–**6** (all metabolites of **1**) were analyzed for complexing potential. Only **1** and **2** formed chromophores absorbing between 450 and 460 nm, the region where these complexes characteristically display maximal absorption of visible light.[15-19]

Figure 1 displays difference spectra obtained for 30 μM **1a** and 30 μM **1b** over a time period of 15 min. The tracings for racemic **1** were essentially identical with those shown for **1b**. The intensity of the difference spectra

(14) J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, **240**, 863 (1965).

(15) J. Werringloer and R. W. Estabrook, *Life Sci.*, **13**, 1319 (1973).
(16) J. C. Kawalek, W. Levin, D. Ryan, and A. Y. H. Lu, *Drug. Metab. Dispos.*, **4**, 190 (1976).
(17) R. C. James and M. R. Franklin, *Biochem. Pharmacol.*, **24**, 835 (1975).
(18) D. Mansuy, P. Beaune, J. C. Chottard, J. F. Bartoli, and P. Gans, *Biochem. Pharmacol.*, **24**, 609 (1975).
(19) J. Jonnson and B. Lindeke, *Acta Pharm. Suec.*, **13**, 313 (1976).
(20) It will be noted that the rates and extents of cytochrome P-450 complex formation for a given amine differ dramatically from one experiment to another. These variations presumably reflect differences in enzyme activites of microsomes isolated from different animals. As reported in Table II, however, replicate analyses using the same microsomal preparation gave reproducible results. The large standard deviation observed for racemic **1** (**1a**,b in Table II) reflects the use of microsomes from different animals in this one case. Even more relevant is the consistency observed for the relative rates and extents of complex formation for the six amines examined in this study. For example, the relative rates for **1b** and racemic **1** always are the same and 3 to 5 times slower than **1a** (compare values in Tables II, IV, and V).

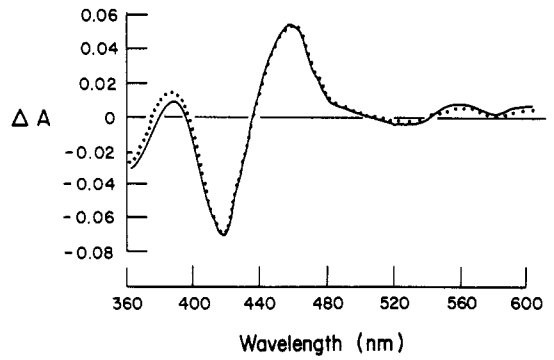


Figure 2. Difference spectra for 30 μM **2a** (···) and 30 μM **2b** (—) obtained immediately after addition of 0.4 mM NADPH.

Table II. Initial Rates and Extents of Cytochrome P-450 Metabolic Intermediate Complex Formation at 30 μM Substrate Concentrations

compd	no. of determinations	lag time,[a] s	initial rates of complex formation[b]	extents of complex formation[c]
1a	3	14	17.1 ± 1.1	78 ± 3
1b	3	136	3.1 ± 0.5	29 ± 6
1ab[d]	3	138	4.8 ± 2.0	30 ± 4[e]
2a	1	0	182	124
2b	1	0	211	161
2ab[d]	3	0	154 ± 18	131 ± 12

[a] The time lapse between the time of addition of NADPH and the first appearance of the 457-nm chromophore. [b] Initial slopes of the absorbance-time curves [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}]. [c] Absorbances [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$] at plateaus of absorbance-time curves. [d] Refers to racemic mixture. [e] These values were obtained from determinations employing microsomes isolated from different animals.

increased with time, the maximum absorption being reached in all three cases by 15 min. Although qualitatively similar, these tracings suggest that **1a** is a better substrate for metabolic intermediate complex formation than either **1b** or racemic **1**. The corresponding data obtained for **2a**, **2b** (Figure 2), and racemic **2** established that, unlike the parent amine, complex formation is insensitive to the chirality of the hydroxylamine. Additionally, although these tracings are qualitatively similar to those obtained with **1**, they indicate that both the rate and extent of complex formation are significantly greater with **2** than with **1**.

In all cases studied, the difference in absorptivity between the reference and sample cell was zero at 500 nm. Therefore, the kinetics of complex formation could be studied conveniently by dual-wavelength spectroscopy in which the difference in absorption of the sample cell at 457 vs. 500 nm is plotted against time. The initial rates (slope of absorbance curve) and maximum extents (maximum absorbance, ΔA_{max}) of complex formation for 30 μM **1a**, **1b**, racemic **1**, **2a**, **2b**, and racemic **2** were determined by this procedure. Based on a previous study with *N*-hydroxyamphetamine (**10**), this substrate concentration was expected to be less than or near the K_m (apparent) for complex formation.[10]

The results (Table II) establish that the cytochrome P-450 complex forms considerably more slowly and to a lesser extent with the parent amine than with the hydroxylamine. A lag time in the appearance of the chromophore was observed with **1a** and **1b**, whereas chromophore formation with **2a**, **2b**, and racemic **2** was essentially instantaneous. These characteristics are consistent with the proposal that amine **1** undergoes microsomal N-hydroxylation prior to formation of the 457-nm absorbing complex.[18,19] Since NADPH is required for chromophore generation with the hydroxylamine as well as the parent amine, further metabolic oxidation of **2** must precede final adduct formation.

Table III. Kinetic Analysis of the Cytochrome P-450 Metabolic Intermediate Complex Formation

compd	no. of determinations	concn range, μM	range of initial rates of complex formation[a]	range of extents of complex formations[b]
1a	6	3.75-120	17.5-19.2	23-72
1b	6	7.5-120	0-2.5	0-15
2a	5	7.5-180	30-144	23-118
2b	4	15-180	66-123	34-104

[a] Initial slopes of the absorbance-time curves [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}]. [b] Absorbances [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$] at plateaus of absorbance-time curves.

These results also demonstrate the stereoselective conversion of the *R* enantiomer **1a** of the parent amine to the adduct-forming species. In this series of experiments, **1a** is oxidized 5.5 times more rapidly and 2.7 times more extensively than the *S* enantiomer **1b**. Since no stereoselectivity is observed with the hydroxylamine isomers, it is likely that N-hydroxylation is the rate-determining step in the overall conversion of **1** to the P-450 complex.

To further examine the characteristics of this complex formation, we conducted a series of experiments in which the rates and extents of complex formation were determined at several substrate concentrations of **1a**, **1b**, **2a**, and **2b**. In some instances the data had to be discarded because of the sudden appearance of a new species which absorbed at 450 nm and which caused a sharp and short-lived increment in the otherwise smooth growth of the 457–500 nm curve. This phenomenon which occured infrequently in our studies has been observed previously[21] and presumably is due to the development of anaerobiasis and the concomitant production of a small amount of carbon monoxide that combines with cytochrome P-450 to produce the typical ferrocarbonyl species absorbing at 450 nm.[22]

The results of these studies are summaried in Table III. The rate of complex formation for the *R* enantiomer **1a** of the parent amine was not concentration dependent. Over a wide concentration range the rates did not dramatically differ and averaged 18 rate units ($\Delta A_{457-500nm} \times 10^4$ nmol P-450^{-1} min^{-1}). It is possible that the apparent zero-order kinetics observed for **1a** reflects a very low K_m for this substrate or that complex formation is not rate limiting. The data, however, clearly establish that at all concentrations studied the *R* enantiomer is a better substrate for complex formation than the S enantiomer. In the concentration range studied, the ratios (*R*/*S*) for the rate and extent of complex formation are minimally seven- and fourfold, respectively. Similar results have been reported for amphetamine (**9**).[17] Unlike the parent amines, the *N*-hydroxy enantiomers **2a** and **2b** displayed comparable kinetic parameters. Again, analogous results have been reported for the enantiomers of *N*-hydroxyamphetamine (**10**).[19]

The kinetic behavior of racemic **1** also was examined. At 30, 150, and 300 μM substrate, the initial rates of

(21) M. Franklin, personal communication.
(22) W. Levin, A. Y. H. Lu, M. Jacobson, R. Kuntman, J. L. Pyer, and P. B. Mc Cay, *Arch. Biochem. Biophys.*, 158, 842 (1973).

Table IV. Comparison of the Initial Rates of Cytochrome P-450 Metabolic Intermediate Complex Formation with 1a, 1b, and Racemic 1

compd	concn, μM	initial rates of complex formation[a]	rate ratios 1a/1b	1a/1ab	1b/1ab
1a	30	5.2			
1b		1.7	3.0	2.9	0.94
1ab[b]		1.8			
1a	150	6.1			
1b		1.5	4.0	3.8	0.94
1ab		1.6			
1a	300	5.5			
1b		1.1	5.0	4.6	0.92
1ab		1.2			

[a] Initial slopes of the absorbance-time curves [$\Delta A_{457-500}$ [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}. [b] Refers to racemic 1.

Table V. Inhibitory Effects of Racemic 2 (30 μM) on the Metabolic Intermediate Complex Formation between Cytochrome P-450 and 1a, 1b, and 1ab[a] (All at 30 μM)

	initial rates[b] and extents[c] of complex formation			
	preincubations with 1a, 1b, or 1ab (30 μM)		following incubations with 2ab (20 μM)	
compd	rate	extent	rate (% inhibn)	extent (% inhibn)
			122 (0)	124 (0)
1a	12.4	51	87 (29)	90 (27)
1b	5.0	27	89 (27)	108 (13)
1ab	5.5	30	85 (30)	109 (12)

[a] Refers to racemic 1. [b] Initial slopes of the absorbance-time curves [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}]. [c] Absorbances [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$] at plateaus of absorbance-time curves.

complex formation for racemic 1 were essentially indistinguishable from those of **1b** (Table IV). The slow rate of complex formation observed with racemic 1 vs. **1a** suggest that **1b** inhibits the metabolic conversion of **1a** to its complex-forming species. A similar inhibitory effect of **1b** on the rate of disappearance of **1a** in rabbit liver microsomal incubations of racemic 1 has been observed.[6] In view of the results obtained in the present study, the inibition by **1b** of the overall rate of metabolism of **1a** may be explained in part by the reduced rate of N-oxidation of **1a** in the presence of **1b**.

Cross Inhibition Studies. Studies originating primarily in Franklin's laboratory have established that complexation of cytochrome P-450 with ligands derived metabolically from amphetamine (**9**) and *N*-hydroxyamphetamine (**10**) leads to inhibition of cytochrome P-450 dependent mixed function oxidase activity.[17] Therefore, we decided to determine if preincubation of the parent amine interfered with microsomal cyclochrome P-450 complex formation following the addition of the hydroxylamine. The inverse experiment involving preincubation of the hydroxylamine and analysis of complex formation following addition of the parent amine also was performed. Since complex formation is insensitive to the chirality of the hydroxylamine, racemic **2** was used in these studies.

Table V summarizes the results of experiments designed to determined if preincubation of racemic 1 or its enantiomers with NADPH-supplemented rabbit liver microsomes leads to impairment of the metabolic oxidation of racemic **2** to its metabolic intermediate complex forming species. Formation of the 457-nm absorbing complex was monitored during the preincubation period, and when this had plateaued (6–8 min) racemic hydroxylamine was added. The initial rates and extents of complex formation were then determined from the $\Delta A_{457-500nm}$ vs. time tracings. The data clearly establish that both the rate and extent of complex formation from racemic **2** are inhibited by racemic 1 and its enantiomers. However, the effects are rather modest and do not appear to be sensitive to the chirality of **1**.

Table VI. Inhibitory Effects of Various Concentrations of 1a on the Metabolic Intermediate Complex Formation between Cytochrome P-450 and Racemic 2 (300 μM)

	initial rates[a] and extents[b] of complex formation			
	preincubation with 1a		following incubation with racemic 2 (300 μM)	
concn of 1a, μM	rate	extent	rate	extent
30	5.2	21	203	131
150	6.1	25	183	116
300	5.8	23	160	105

[a] Initial slopes of the absorbance-time curves [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}]. [b] Absorbances [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ at plateaus of absorbance-time curves.

The effect of the concentration of **1a** on complex formation from racemic **2** was examined by preincubating amine **1a** at 30, 150, and 300 μM and examining complex formation with 300 μM racemic **2**. This concentration of racemic **2** was chosen since it produced maximal complex formation. The results, which are summarized in Table VI, show that the inhibition of racemic **2** complex formation by **1a** is concentration dependent even though complex formation from **1a** itself is concentration independent. This experiment again illustrates the modest effect the parent amine has on the oxidation of racemic **2**.

A second experiment involving preincubation with varying concentrations of racemic **2**, followed by addition of 30 μM **1a**, was undertaken in an effort to determine the inhibitory effect of metabolic intermediate complex formation on the N-oxidation of **1a**. In an attempt to assess residual enzymatic activity, complex formation was monitored following a second addition of racemic **2** (30 μM).

The results of these studies are summarized in Table VII. The principal finding is that relatively low concentrations of racemic **2** dramatically inhibit the NADPH-dependent conversion of **1a** to the ligand that binds to cytochrome P-450. The capacity of the mixed function oxidase system to oxidize racemic **2** to this ligand after exposure to both racemic **2** and **1a** is only partially impaired. For example, following the preincubation of 15 μM racemic **2**, metabolic oxidation of **1a** to the ligand binding to cytochrome P-450 is completely inhibited, whereas the initial rate and maximum extent of complex formation following the second addition of racemic **2** is near 50% of the values achieved when racemic **2** is incubated alone with fresh microsomes. These results are consistent with the proposal that metabolic intermediate complex formation of cytochrome P-450 with primary amines such as 1 requires two oxidative steps, the first step being N-hydroxylation, and that complex formation inhibits the NADPH-dependent N-hydroxylation step. It would appear that this inhibition is selective in that racemic **2** remains an effective substrate for complex formation even though the N-hydroxylation reaction is completely inhibited. Perhaps different forms or different catalytic sites of cytochrome P-450 are involved in these transformations.

Table VII. Inhibitory Effects of Various Concentrations of Racemic 2 on the Metabolic Intermediate Complex Formation between Cytochrome P-450 and 1a (30 μM)

	initial rates[a] and extents[b] of complex formation					
	preincubation with 2ab		following incubation with 30 μM 1a		following incubation with 30 μM 2ab	
concn of 2ab,[c] μM	rate	extent	rate (% inhibn)	extent (% inhibn)	rate (% inhibn)	extent (% inhibn)
0			46 (0)	76 (0)	124 (30)	95 (30)
5	52	39	9 (80)	43 (43)	107 (39)	85 (39)
15	131	97	0 (100)	0 (100)	86 (51)	75 (46)
30	177	139	0 (100)	0 (100)	45 (74)	37 (73)
30	176	135			50 (72)	46 (66)

[a] Initial slopes of the absorbance-time curves [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$ min^{-1}]. [b] Absorbances [$\Delta A_{457-500nm} \times 10^4$ (nmol of cytochrome P-450)$^{-1}$] at plateaus of absorbance-time curves. [c] Refers to racemic 2.

Summary

The results presented in this paper strongly support the hypothesis[19] that metabolic intermediate complex formation of 1-phenyl-2-aminopropanes proceeds first by conversion of the parent amine to the corresponding *N*-hydroxy derivative, followed by further NADPH-dependent oxidation of the hydroxylamine to a species which forms a tight complex with cytochrome P-450. Since complex formation appears to be insensitive to the chirality of the hydroxylamine 2, the enantioselective formation of the complex observed with (*R*)-1 must be determined primarily by the N-hydroxylation step.

Cross inhibition studies showed that complexation of cytochrome P-450 with even low concentrations of the hydroxylamine dramatically blocks the microsomal conversion of the parent amine to the complex-forming species. Since the complex-forming capacity of the microsomes is not saturated, it seems likely that cytochrome P-450 complexation selectively inhibits the NADPH-dependent N-hydroxylation of 1. This conclusion implies that N-hydroxylation of 1 is a suicide mixed function oxidase pathway.

Earlier kinetic studies on the overall microsomal metabolism of racemic 1 and its enantiomers showed that the metabolism of (*R*)-1 is significantly inhibited by (*S*)-1.[6] Results obtained in the present study indicate a similar enantiomeric interaction. The rates of complex formation observed with racemic 1 and (*S*)-1 are essentially identical and are considerably slower than the rate observed with (*R*)-1. It seems likely, therefore, that (*S*)-1 inhibits the N-hydroxylation of (*R*)-1. Since N-hydroxylation appears to be a suicide pathway, this interaction may lead to an alteration of the relative extents of the various metabolic pathways which 1 undergoes. Additional metabolic studies employing the individual isomers of 1 should lead to a better understanding of the potential metabolic consequences of such enantiomeric interactions.

Experimental Section

Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. NMR spectra were taken on either a Varian Associates A-60A instrument or a Perkin-Elmer 12B instrument, and chemical shifts are reported in parts per million (δ) downfield relative to Me_4Si ($CDCl_3$). CIMS analyses were obtained using an AEI MS 902 instrument modified for CIMS using isobutane (0.5 torr) as reactant gas. Spectral studies were carried out on an Aminco DW-2 recording spectrophotometer. Elemental analyses were performed by the Microanalytical Laboratory of the University of California, Berkeley.

Microsomal Preparations. Hepatic microsomes were prepared from male Dutch rabbits 6–12 months old and weighing 1.5–2.5 kg. All animals were fasted 24 h prior to sacrifice to reduce liver glycogen. They were then sacrificed by a blow to the neck and decapitation, and their livers were perfused in situ with isotonic KCl in 0.01 M phosphate buffer (pH 7.4) via the portal vein until the lobes were free of blood. The liver lobes were removed and rinsed in the KCl buffer solution. Liver was then weighed, scissor minced, and homogenized in 2 volumes of ice-cold KCl buffer solution. The homogenates were centrifuged at 10000*g* for 20 min in a Sorval RCZ-B centrifuge held at 4 °C, and the supernatant fractions were further centrifuged at 100000*g* for 1 h, in a Spinco Model L centrifuge held at 4 °C to yield microsomal pellets. To ensure complete removal of hemoglobin, the pellets were resuspended to about half the original supernatant volume with the KCl buffer solution, and the suspension was again centrifuged at 100000*g* for 1 h. The microsomal pellets were then suspended in 50 mM Tris-Cl buffer containing 150 mM KCl and 10 mM $MgSO_4$. The resulting suspensions, containing 15–25 mg of microsomal protein/mL, were kept on ice and fractions diluted as needed. Protein determinations were made by the method of Sutherland.[23]

Spectrophotometric observations were made using microsomes prepared as above and diluted, with the addition of 0.1 mM EDTA, to 2 mg of protein/mL. Cytochrome P-450 concentrations were determined as the reduced-CO complex, assuming in all cases an extinction coefficient of 91 mM^{-1} cm^{-1}. The spectral characterization of the cytochrome P-450 complexes was determined by difference spectroscopy, and the rates of formation of these complexes were determined by means of the dual-wavelength mode (457 vs. 500 nm). Binding studies were carried out at ambient temperature (ca. 31 °C), while the enzymatic reactions were carried out at 37 °C using a temperature-regulated Aminco DW-2 spectrophotometer. NADPH was obtained from Sigma Chemical Co. The synthesis, resolution, and absolute configuration assignments of 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (1) have been reported previously.[4]

(*RS*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-[(benzoyloxy)amino]propane (7). To a solution of 1.26 g (5.20 mmol) of benzoyl peroxide, previously purified by recrystallization from chloroform, in 15 mL of benzene was added dropwise a solution of 2.17 g of amine 1 (10.3 mmol) in 15 mL of benzene. The reaction mixture was stirred magnetically under nitrogen and kept at room temperature with a water bath. The reaction was complete in 2 h. After the mixture cooled to 10 °C, the precipitated benzoate salt of 1 was removed by suction filtration. The filtrate was taken to dryness under vacuum, and the residue was digested with 30 mL of hexane. The amide side product, 1-(2,5-dimethoxy-4-methylphenyl)-2-benzamidopropane (8), identified by comparison with an authentic sample,[4] was removed by suction filtration. The solvent was removed under vacuum and the viscous oil was purified by column chromatography (45 g of silica gel, 1% ethyl acetate in hexane). Pure 7 (1.18 g, 70%) was recovered as an oil, which subsequently crystallized upon standing in the refrigerator: mp 30–32 °C; NMR ($CDCl_3$) δ 1.24 (d, 3, J = 3.3 Hz, $CHCH_3$), 2.2 (s, 3, Ar CH_3), 2.82 (m, 2, CH_2), 3.53 (m, 1, $CHCH_3$), 3.72 (s, 3, OCH_3), 3.74 (s, 3, OCH_3), 6.64 (s, 1, Ar H), 6.66 (s, 1, Ar H), 7.41 (m, 4, NH, Ar H_3), 7.98 (m, 2, Ar H_2); CIMS, *m/e* (relative intensity) 330 (100, MH^+), 108 (42), 165 (24), 123 (21). Anal. ($C_{19}H_{23}NO_4$) C, H, N.

(*R*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-[(benzoyloxy)amino]propane (7a). To a solution of 0.282 g of benzoyl peroxide (1.65 mmol) in 5 mL of benzene in a 20-mL round-bottomed flask

(23) E. W. Sutherland, C. F. Corti, R. Haynes, and N. S. Olsen, *J. Biol. Chem.*, **180**, 825 (1949).

was added dropwise a solution of 0.487 g of **1a** (2.33 mmol) in 5 mL of benzene. The reaction was carried out, monitored, and worked up as described above for racemic **7**. The crude product **7a** was purified by column chromatography as above to yield 0.228 g (38%) of pure **7a**.

(*S*)-2-(2,5-Dimethoxy-4-methylphenyl)-2-[(benzoyloxy)amino]propane (7b). To a solution of 1.26 g of benzoyl peroxide (5.20 mmol) in 15 mL of benzene in a 100-mL round-bottomed flask was added dropwise a solution of 2.17 g of **1b** (10.3 mmol) in 15 mL of benzene. The crude product was purified by column chromatography as above to yield 1.32 g (39%) of pure **7b**.

(*RS*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-(hydroxyamino)propane (2ab). To 0.515 g of racemic **7** (3.58 mmol) was added 3.6 mL of 95% ethanol and 7.2 mL of concentrated HCl, both of which had been purged with nitrogen, in a 10-mL round-bottomed flask. The solution was magnetically stirred and heated under reflux under nitrogen. The reaction was complete after 135 min. The ethanol was removed under vacuum and the residue was taken up in 5 mL of deionized water. The solution was then lyophyllized overnight to remove water and HCl. This left a dark purple powder containing crude **2** as the hydrochloride salt. Recrystallization from ethanol/ether gave pure **2** (41%), mp 128–130 °C (lit.[5] mp 126–128 °C).

(*R*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-(hydroxyamino)propane (2a). To 0.29 g (0.882 mmol) of **7a** in a 10-mL round-bottomed flask was added 1 mL of 95% ethanol and 2 mL of concentrated HCl, both of which had been purged with nitrogen. The solution was magnetically stirred and heated under reflux and nitrogen. The reaction was allowed to proceed for 180 min and was worked up as described above. The crude product **2a** was recrystallized from ethanol/ether to give 0.049 g (21%) of pure **2a**: mp 137.5–139 °C.

(*S*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-(hydroxyamino)propane (2b). To 1.18 g of **7b** (3.58 mmol) was added 3.6 mL of 95% ethanol and 7.2 mL of concentrated HCl, both of which had been purged with nitrogen, in a 25-mL round-bottom flask. The solution was magnetically stirred and heated under reflux under nitrogen. The reaction was allowed to proceed for 150 min and was worked up as described above. The crude **2b** was recrystallized from ethanol/ether to give 0.352 g (38% of pure **2b**: mp 137.5–139 °C. anal. ($C_{12}H_{20}NO_3Cl$) C, H, N.

Acknowledgment. This research was supported by Public Health Service Research Grant MH 21219.

Puromycin Analogues. Effect of Aryl-Substituted Puromycin Analogues on the Ribosomal Peptidyltransferase Reaction

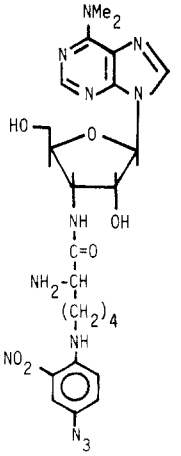
Heejoo Lee, Kei-Lai Fong, and Robert Vince*

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.
Received July 25, 1980

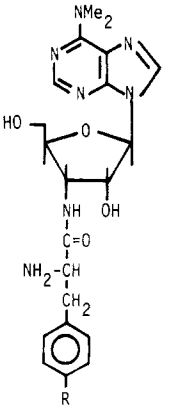
A series of ortho- and para-substituted L-phenylalanylpuromycin analogues were synthesized and evaluated as substrates for the peptidyltransferase reaction of *Escherichia coli* ribosomes. Kinetic results reveal that substitution of the *p*-methoxy group of the puromycin molecule alters the peptidyltransferase activity of the molecule with the following decreasing order of substrate efficiencies: *p*-NH_2 > *p*-$NHCOCH_3$ > *p*-NO_2 = *p*-$NHCO(CH_2)_2CH_3$ > *p*-$NHCOCH_2Br$. However, the inability of the ribosome to tolerate a nitro group at the ortho position of the phenylalanine ring precluded the use of the photosensitive puromycin analogue, 2-nitro-4-azidophenylalanylpuromycin aminonucleoside (**7a**), as a photoaffinity label for the peptidyltransferase site.

Numerous antibiotics have been shown to bind to bacterial and/or eucaryotic ribosomes and inhibit protein synthesis. The peptide chains of proteins are synthesized on ribosomes by a series of reactions culminating in peptide bond formation catalyzed by peptidyltransferase.[1,2] Current models of the active center of peptidyltransferase invoke a P site which binds the CCA terminus of peptidyl-tRNA and an A site which binds the CCA terminus of aminoacyl-tRNA.[3] The antitumor antibiotic puromycin inhibits protein synthesis by substituting for the incoming coded aminoacyl-tRNA at the A site and serving as an acceptor of the nascent peptide chain of ribosome-bound peptidyl-tRNA.[3] For this reason, puromycin has been used extensively in the investigation of the peptidyltransferase site.[4,5]

Chart I



Nap-Lys-PAN



p-Azidopuromycin R = N_3

Puromycin R = OCH_3

Several approaches have recently been developed to label the A-site protein(s) of peptidyltransferase.[5a,6-10] In

(1) R. E. Monro, *J. Mol. Biol.*, **26**, 147 (1967).
(2) B. E. H. Maden, R. R. Traut, and R. E. Monro, *J. Mol. Biol.*, **35**, 333 (1968).
(3) (a) R. E. Monro, T. Staehlin, T. Celma, and D. Vazquez, *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 357 (1969); (b) A. J. Morris and R. S. Schweet, *Biochim. Biophys. Acta*, **47**, 415 (1961); (c) M. Rabinovitz and J. M. Fisher, *J. Biol. Chem.*, **237**, 477 (1962); (d) D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.*, **51**, 585 (1964).
(4) R. J. Harris and S. Pestka, in "Molecular Mechanisms of Protein Biosynthesis", H. Weissbach and S. Pestka, Eds., Academic Press, New York, 1977, pp 413–442.
(5) (a) R. Vince, J. Brownell, and K. L. L. Fong, *Biochemistry*, **17**, 5489 (1978); (b) K. L. L. Fong and R. Vince, *J. Med. Chem.*, **21**, 792 (1978); (c) R. Vince and K. L. L. Fong, *Biochem. Biophys. Res. Commun.*, **81**, 559 (1978).

0022-2623/81/1824-0304$01.00/0